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Purification and characterization of 4-methylmuconolactone methyl-isomerase, a novel enzyme of the modified 3-oxoadipate pathway in nocardioform actinomycetes

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The novel enzyme 4-methyl-2-enelactone methyl-isomerase was detected in, and purified to electrophoretic homogeneity from, *p*-toluate-grown cells of *Rhodococcus rhodocrous* N75, a nocardioform actinomycete. The enzyme was very thermostable and had a native M_r of 75500; as the monomer had an M_r of 17000, the enzyme is probably tetrameric. The new isomerase is highly specific with respect to its lactone substrate, only accepting (+)-(4*S*)-4-methylmuconolactone (4-carboxymethyl-4-methylbut-2-en-1,4-olide), and the putative isomerization reaction intermediate 1-methylbislactone {(−)-1-methyl-3,7-dioxo-2,6-dioxabicyclo-[3.3.0]octane} as substrates, and yielding (−)-(4*S*)-3-methylmuconolactone (4-carboxymethyl-3-methylbut-2-en-1,4-olide) as product. Some other lactone analogues acted as competitive inhibitors. Our data suggest that the isomerization does not involve actual methyl migration, but proceeds via the 1-methybislactone.

INTRODUCTION

The *ortho*-fission pathway for catabolism of 3- and 4-methyl-substituted mononuclear aromatics is generally precluded in most bacteria, because the methylmuconolactone intermediate formed at the lactonization step bears the methyl substituent at C-4 and is thus unable to undergo the classical muconolactone isomerase reaction (Stanier & Ornston, 1973). The intermediate 4-methylmuconolactone [4-carboxymethyl-4-methylbut-2-en-1,4-olide; trivially, 4-methyl-2-enelactone (**I**)] accumulates as a 'dead-end' metabolite with such organisms (Catelani *et al.*, 1971).

The nocardioform actinomycetes, however, have evolved a modified 3-oxoadipate pathway to catabolize such methyl-substituted aromatics (Bruce & Cain, 1988; Cain *et al.*, 1989). They circumvent this problem by isomerizing 4-methyl-2-enelactone to 3-methylmuconolactone [4-carboxymethyl-3-methylbut-2-en-1,4-olide, trivially, 3-methyl-2-enelactone (**II**)], which is further degraded to 4-methyl-3-oxoadipate in a manner presumably analogous to that involved in the conversion of (+)-muconolactone to 3-oxoadipate (Stanier & Ornston, 1973).

In the present investigation we report the purification and characterization of the novel enzyme, 4-methyl-2-enelactone methyl-isomerase, from the nocardioform actinomycete *Rhodococcus rhodocrous* N75. Evidence is also presented for the possible participation of 1-methyl-3,7-dioxo-2,6-dioxabicyclo[3.3.0]octane [trivially, 1-methyl-bislactone (**Va**)] as a reaction intermediate in the conversion of 4-methyl-2-enelactone into 3-methyl-2-enelactone.

MATERIALS AND METHODS

The organism used in this investigation was *R. rhodocrous* N75 (LA1069), originally supplied by Dr. M. Goodfellow, Department of Microbiology, University of Newcastle upon Tyne, Newcastle upon Tyne, U.K., as *Gordona rubra* and incorrectly named at *R. ruber* by Bruce & Cain (1988). Conditions for bulk growth of the bacteria (20-litre batches, vigorously sparged with sterile air) and preparations of cell-free extracts were described by Miller (1981). These cultures gave extracts with considerably higher activities of the isomerase (1.5–3.6 units·mg of protein^{-1}) than those obtained from 750 ml cultures in 2-litre flasks incubated at 30 °C in an orbital shaker at 180 rev.·min^{-1} (300–500 munits·mg of protein^{-1}).

Purification of 4-methyl-2-enelactone methyl-isomerase

All procedures were performed at 4 °C unless a different temperature is specified, and all centrifugations were run at 16000 *g* for 20 min.

(i) Preparation of cell-free extract. Crude extract was prepared from 27 g (wet weight) of frozen *p*-toluate-grown cells that were thawed at 4 °C before extraction. The cells were resuspended in buffer A (50 mM-Tris/HCl, pH 7.0, containing 0.5 mM-dithiothreitol) at a concentration of 0.5 g/ml (wet weight) and were disrupted by 3 min ultrasonication while cooled in melting ice. The sonicated cell suspension was centrifuged to remove the cell debris.

(ii) Streptomycin sulphate treatment. A neutralized 10% (w/v) streptomycin sulphate solution was added

Abbreviation used: SDS/PAGE, SDS/polyacrylamide-gel electrophoresis.

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dropwise with constant stirring to 87 ml of crude cell extract until 0.1 ml of streptomycin sulphate had been added per 1 ml of cell extract. After stirring at 4 °C for 5 min, the nucleic acid precipitate was removed by centrifugation.

(iii) Heat treatment. In crude extracts prepared from cells grown on *p*-toluate as carbon source, the enzyme was observed to be very resistant to heat denaturation. The supernatant from the streptomycin sulphate step was heated at 65±0.1 °C for 15 min in a constant-temperature water bath. Precipitated protein was removed by centrifugation.

(iv) DEAE-Sephacel ion-exchange chromatography. The heat-treated preparation was applied to a DEAE-Sephacel column (2.5 cm × 14 cm) that had been equilibrated with buffer A. After adsorption on to the column, the sample was washed extensively with buffer A containing 0.1 M-NaCl until no further absorbance at 280 nm was evident in the eluate (approx. 450 ml), then the isomerase was eluted with 400 ml of buffer A containing NaCl in a linear gradient running from 0.1 M to 0.3 M. Fractions of 8 ml were collected at a flow rate of 25 ml/h. The enzyme was eluted at approx. 0.17 M-NaCl.

(v) AcA-34 gel filtration. Fractions from ion-exchange-chromatographic procedure containing the highest isomerase activity were combined and concentrated to 2.5 ml by ultrafiltration. The concentrate was applied to an Ultrogel AcA-34 column (1.5 cm × 100 cm) that had previously been equilibrated with buffer B (10 mM-potassium phosphate, pH 7.0, containing 0.5 mM-dithiothreitol). The flow rate was maintained at 4 ml/h, and fractions of 3 ml were collected.

SDS/polyacrylamide-gel electrophoresis (SDS/PAGE)

SDS/PAGE was performed by the method of Laemmli (1970) on 1 mm-thick vertical slab gels (20.5 cm × 16 cm), containing 12.5% (w/v) acrylamide in the resolving gels. Electrophoresis was performed at a current of 30 mA for 5–6 h, or until the Bromophenol Blue tracking dye reached the bottom of the gel. M_r determinations of the purified enzyme were obtained by using a Sigma MW-SDS-70L M_r calibration kit. Protein was detected by staining the gels for 6 h with Coomassie Brilliant Blue R250 dissolved in methanol/water/acetic acid (5:5:1, by vol.). Gels were diffusion destained by repeated washing in the above solvent mixture.

Assay procedures for 4-methyl-2-enelactone methylisomerase

The first procedure depended upon separation and quantitative determination of the two lactone isomers by reverse-phase h.p.l.c., in samples taken throughout the incubation period (Bruce & Cain, 1988). This technique clearly separated mixtures of lactones **I**, **II**, **III**, **IV** and **XIII** (Fig. 1) and all of these from the acids **XIV** and **XV** (Table 2 below). The second procedure for measuring the isomerization reaction, suitable for use with purified enzyme preparations, depended on monitoring the increase in absorbance at 225 nm resulting from the conversion of 4-methyl-2-enelactone (ϵ 2670 litre·mol^{-1}·cm^{-1}) into 3-methyl-2-enelactone (ϵ 6300 litre·mol^{-1}·cm^{-1}). The difference in absorbance maxima between these two compounds in phosphate buffer, pH 7.0, results in 3-methyl-2-enelactone having a greater absorption coefficient than the 4-methyl isomer at 225 nm ($\Delta\epsilon$ 3630 litre·mol^{-1}·cm^{-1}). The assay mixtures contained, in 1 ml of 50 mM-potassium phosphate buffer, pH 7.0: 4-methyl-2-enelactone, 0.3 μmol; and isomerase enzyme, 5–50 μl containing approx. 0.01–0.05 units of enzyme. The cuvette contents were incubated at 30 °C, and absorbance changes at 225 nm were amplified by scale expansion of the chart recorder where appropriate. The unit of activity is the amount of enzyme necessary to convert 1 μmol of the 4-methyl-2-enelactone into its isomer/min at 30 °C.

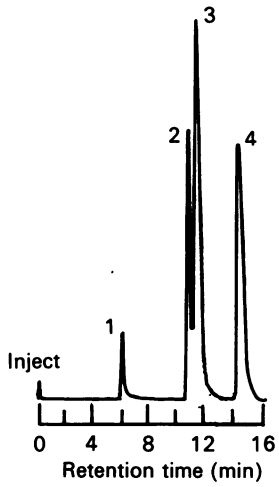


Fig. 1. Separation of lactones by h.p.l.c.

The mixture was resolved on a Spherisorb ODS reverse-phase column (0.46 cm × 25 cm) with a solvent system of aq. 6% (v/v) methanol containing conc. phosphoric acid (1 g·l^{-1}) at a flow rate of 1.5 ml·min^{-1}. Absorbance was monitored at 215 nm. 1, Muconolactone; 2, 4-methyl-2-enelactone; 3, 3-methyl-2-enelactone; 4, 2-methyl-2-enelactone.

Chemicals

(+)-4-Methyl-2-enelactone (**I**) and 2-methyl-2-enelactone (**III**) were obtained from culture filtrates of *Pseudomonas* B13 previously grown with 3-chlorobenzoate and then incubated with *p*-toluate and *m*-toluate respectively (Knackmuss *et al.*, 1976). The lactones were recrystallized from di-isopropylether/benzene and their structures confirmed by m.s. and proton n.m.r. (−)-1-Methylbislactone [(−)-1-methyl-3,7-dioxo-2,6-dioxabicyclo[3.3.0]octane] (**Va**) was synthesized from 4-methyl-2-enelactone as described by Catelani *et al.* (1971) and crystallized from diethyl ether, m.p. 108 °C. (−)-1-Methyl-8-bromobislactone (**Vb**) was produced by treating (**II**) with bromine and aq. $NaHCO_3$; its configuration was established by X-ray analysis (Cain *et al.*, 1989). 3-Methyl-*cis*,*cis*-muconate (**XIV**) was prepared biologically as described by Bruce & Cain (1988). 3-Carboxy-*cis*,*cis*-muconate (trisodium salt) was synthesized from vanillin by the method of Ainsworth & Kirby (1968) and (−)-3-carboxymuconolactone prepared from it with extracts of a hydrolase-negative mutant of *Aspergillus niger* (Ahlquist, 1977). (+)-Muconolactone (**IV**), m.p. 74–75 °C, was also obtained from the culture filtrates of a muconolactone isomerase-negative mutant of *A. niger* incubated with 2 mM-catechol from which the lactone accumulated almost quantitatively (Ahlquist, 1977). The muconic

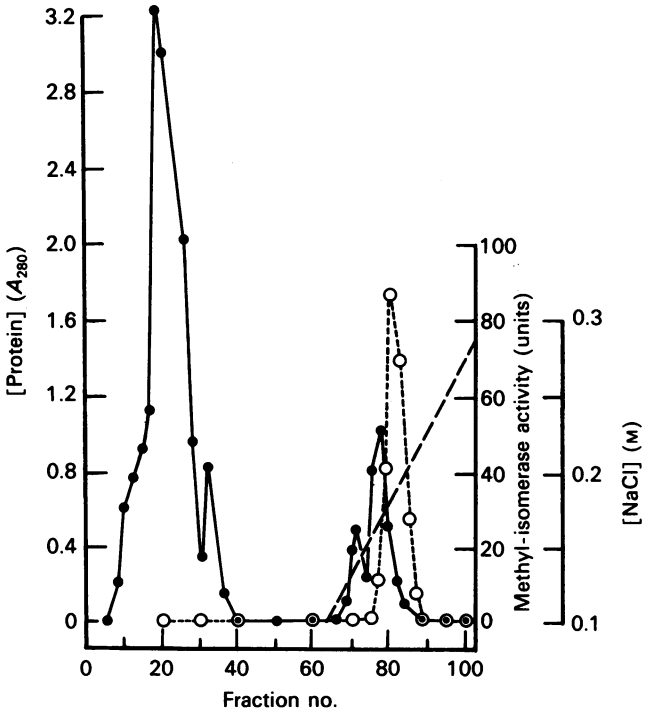


Fig. 2. DEAE-Sephacel chromatography of 4-methyl-2-enelactone methyl-isomerase

The enzyme was eluted with a 400 ml linear gradient (0.1–0.3 M-NaCl in buffer A) (——) at a flow rate of 25 ml/h, 8 ml fractions being collected. Each fraction was assayed for isomerase activity (○) and protein content (●).

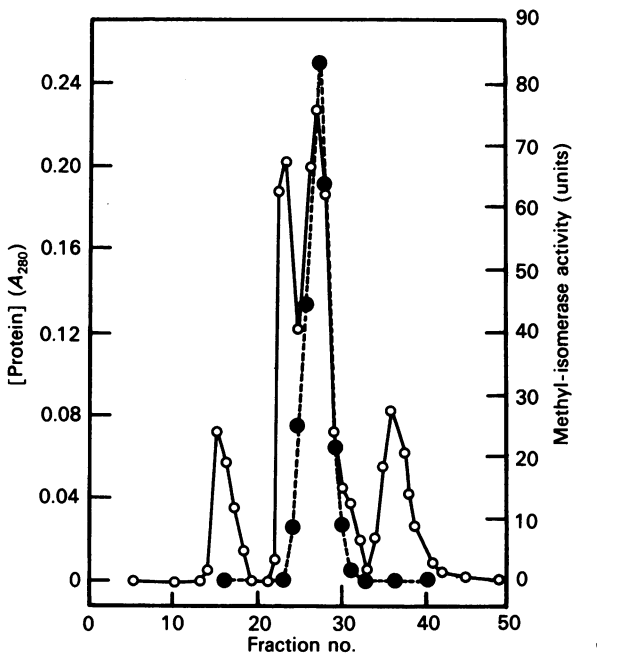


Fig. 3. AcA-34 gel-filtration chromatography of 4-methyl-2-enelactone methyl-isomerase

The enzyme was eluted with buffer B at a flow rate of 4 ml/h, 3 ml fractions being collected. Each fraction was assayed for isomerase activity (●) and protein content (○).

bislactone (3,7-dioxo-2,6-dioxabicyclo[3.3.0]octane) (**VI**) was derived from *cis,cis*-muconic acid as described by Elvidge *et al.* (1950). (−)-3-Methyl-2-enelactone (**II**) was prepared biologically from 4-methylcatechol according to the method of Miller (1981), using crude extracts from cells of *R. rhodocrous* grown at the expense of either 10 mM-*p*-toluate or benzoate. The racemic lactone (**II**) was prepared chemically by H_2SO_4 cleavage of 4-methyl-2-nitrophenol (Elvidge *et al.*, 1951) and subsequently resolved by Dr. G. V. Rao (Department of Chemistry, University of Glasgow, Glasgow, Scotland, U.K.) into its (+)- and (−)-isomers by repeated fractional crystallization of the (*S*)-1-phenylethylamine salts (Cain *et al.*, 1989).

RESULTS

In crude extracts prepared from cells grown on *p*-toluate in shaken-flask cultures, the 4-methyl-2-enelactone methyl-isomerase was present at activities of 350–450 munits·mg of protein^{-1}, some 20-fold greater than that found in benzoate-grown and 75-fold greater than that found in glucose- or succinate-grown cells. In air-sparged bulk cultures on *p*-toluate, the isomerase activities of extracts were considerably elevated (up to 10-fold) above those originating from shaken-flask cultures. The activity was located in the soluble fraction of the

Table 1. Purification of the 4-methyl-2-enelactone methyl-isomerase from *R. rhodocrous*

The starting material was 27 g of cell paste. Full experimental details are given in the Materials and methods section.

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units·mg^{-1})	Recovery of activity (%)	Purification factor
Crude extract	87	1365	374	3.6	100	1
Streptomycin sulphate	100	1310	346	3.9	96	1.1
Heat treatment	100	1065	25	42.6	78	11.6
Anion-exchange chromatography on DEAE-Sephacel	2.5	458	1.25	366	34	100
Gel-filtration on Ultrogel AcA-34	2.3	314	0.4	785	23	215

cellular extracts and was very resistant to heat denaturation. The enzyme, when heated to 70 °C for 15 min, lost only 20% of its activity, although inactivation occurred within 30 min at 80 °C. This thermostability of the catabolic isomerase provided an effective early step in purification. The results of the subsequent protein separation by DEAE-Sephacel chromatography are shown in Fig. 2. Enzyme activity was eluted with approx. 0.17 M-NaCl. Because the enzyme was bound at NaCl concentrations below this, a substantial amount of contaminating protein was removed by the initial washing with buffer containing 0.1 M-NaCl. The protein elution profile for the AcA-34 chromatography step is illustrated in Fig. 3; small amounts of protein emerged before and after the two main protein peaks, the second of which was coincident with a single peak of 4-methyl-2-enelactone methylisomerase activity. The initial two fractions on the ascending side of the activity peak were omitted, the remaining fractions (25–29) containing the highest enzyme activity (Fig. 3) were pooled and concentrated to 1 ml by ultrafiltration. A summary of the final scheme adopted for the purification of the enzyme is presented in Table 1. Samples (12.5 μg of protein) of the ultrafiltrate from the final step were homogeneous by SDS/PAGE and by h.p.l.c. on a Zorbax G-F 250 gel-permeation column (0.94 cm × 25 cm) (du Pont, Wilmington, DE, U.S.A.), from which the enzyme was eluted at 9.69 min in 10 mM-phosphate buffer, pH 7.0, at a flow rate of 0.75 ml·min^{-1}.

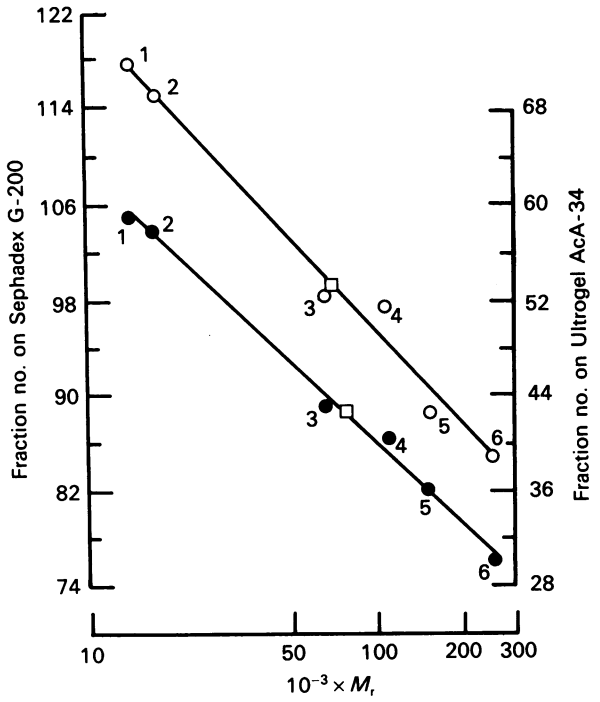


Fig. 4. M_r of 4-methyl-2-enelactone isomerase by gel-filtration chromatography

Purified enzyme (□) was loaded on to columns of AcA-34 (○) or Sephadex G-200 (●), together with a solution of standard marker proteins: 1, cytochrome *c*; 2, myoglobin; 3, bovine serum albumin, 4, hexokinase; 5, alcohol dehydrogenase; and 6, catalase. The activity eluted in the position corresponding to M_r values of 72000 and 79000 from the Ultrogel AcA-34 and Sephadex G-200 columns respectively.

pH optimum

The effect of pH on enzyme activity over the range 6–9 showed an optimum activity at pH 7.0 in potassium phosphate buffer. Decrease of activity was rapid at pH values above 8.5.

M_r

The M_r of the native enzyme was determined by the method of Andrews (1964) from independent measurements on columns of Sephadex G-200 (1.5 cm × 100 cm) and Ultrogel AcA-34 (1.5 cm × 100 cm) calibrated with marker proteins (see Fig. 4). After both columns had been equilibrated with 50 mM-potassium phosphate buffer, pH 7.0, a solution containing purified enzyme was applied to the bed surface of the two gel-filtration columns and eluted with the equilibration buffer at a flow rate of 4 ml/h, 1.5 ml fractions being collected from

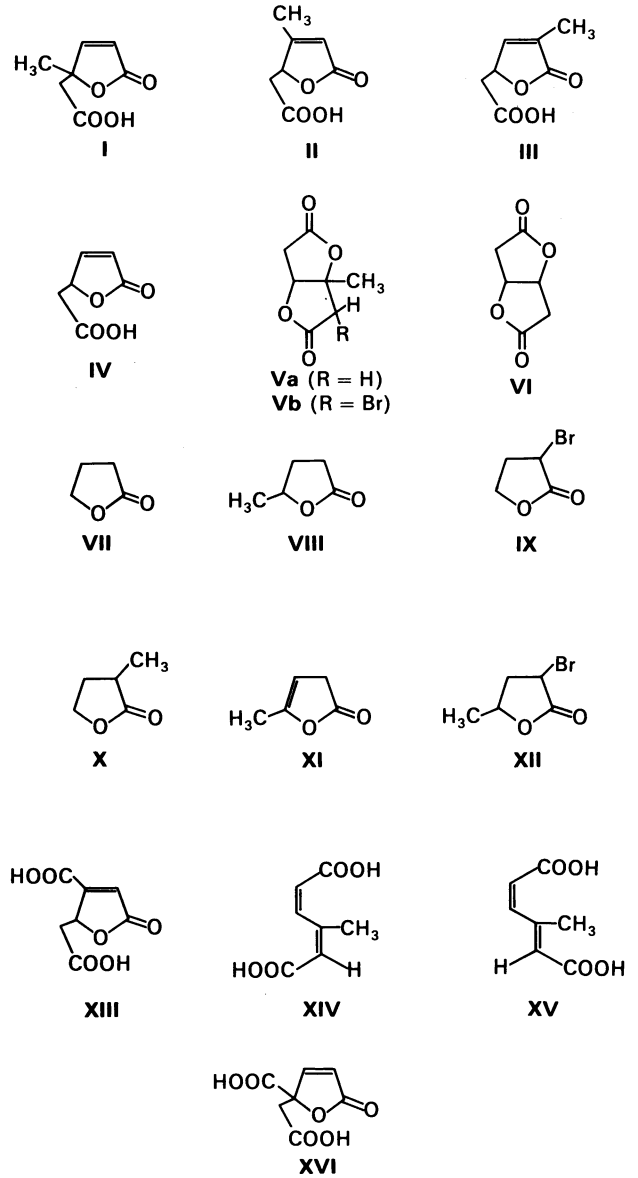


Fig. 5. Analogues used in substrate specificity and inhibitor studies

For the nomenclature of the analogue substrates, see Table 2.

Table 2. Activities of analogues of 4-methylmuconolactone as substrates or inhibitors

Enzyme activity was assayed by h.p.l.c. using 0.3 μg of enzyme and with the analogue substrates at a concentration of 0.3 mM. In a parallel series the analogue was tested as an inhibitor at a concentration of 0.6 mM in the presence of 0.3 mM-4-methyl-2-enelactone. Activities are relative to that determined with 0.3 mM-4-methyl-2-enelactone alone (0.06 μmol·min^{-1}). Structures of the analogues are shown in Fig. 4. Values in parentheses (column 4) are the K_i values (mM) of the inhibitor.

Compound		Relative rates of activity (%)	
No.	Name	Compound alone	Compound with 4-methylmuconolactone
I	(+)-4-Methylmuconolactone	100	100
II	(−)-3-Methylmuconolactone	0	100
IIa	(+)-3-Methylmuconolactone	0	100
III	2-Methylmuconolactone	0	97
IV	(+)-Muconolactone	NM*	65 (0.48)
Va	(−)-1-Methyl-bislactone	221	−†
Vb	(−)-1-Methyl-8-bromobislactone	6‡	84‡
VI	Muconic bislactone	337	124
VII	Butyrolactone (butan-1,4-olide)	NM*	100
VIII	4-Methylbutan-1,4-olide	0	75 (0.78)
IX	2-Bromobutan-1,4-olide	0	78
X	2-Methylbutan-1,4-olide	0	76 (0.80)
XI	4-Methylbut-3-en-1,4-olide (angelicalactone)	0	80
XII	2-Bromo-4-methylbutan-1,4-olide	0	88
XIII	3-Carboxymuconolactone	0	92
XIV	3-Methyl-*cis,cis*-muconate	0§	90
XV	3-Methyl-*cis,trans*-muconate	0§	92

* NM, not measurable as a substrate because it lacks a methyl or other substituent, so the substrate and the putative product are identical.

† Not determined; product identical with that of the substrate.

‡ Very rapidly hydrolysed non-enzymically to 2-bromo-3-methylmuconolactone (see the text); these values probably represent the effects of the hydrolysis product.

§ Also assayed for spectral change resulting from the addition of purified methyl-isomerase to the assay mixture; spectra were scanned initially, immediately after adding purified enzyme and finally after 10 min; none of the spectra showed significant change from the first scan.

the Sephadex G-200 column and 1 ml fractions from the AcA-34 column. The elution volumes of 4-methyl-2-enelactone methyl-isomerase corresponded to M_r values of 79000 and 72000 from the Sephadex G-200 and AcA-34 columns respectively (Fig. 4).

Purified isomerase subjected to SDS/PAGE yielded a single distinct band with an M_r of approx. 17000, as determined from its mobility relative to that of the standard proteins. Comparison of the mean native M_r and the subunit M_r indicated that the isomerase was a possible tetramer composed of identical subunits.

Kinetic properties of the 4-methyl-2-enelactone methyl-isomerase

The conversion of the (+)-4-methyl-2-enelactone into its (−)-3-methyl isomer by the purified enzyme was unidirectional and quantitative; the product was rigorously characterized and was identical in spectral, physical and chemical properties with the compound described by Miller (1981), Pieper *et al.* (1985) and Bruce & Cain (1988). Even with high concentrations of the 3-methyl-2-enelactone product and up to 20-fold the amount of enzyme used in the routine assays, no reversal of the isomerization was demonstrated.

Initial rates of isomerization of 4-methyl-2-enelactone were determined spectrophotometrically at 225 nm using reaction mixtures containing 50 mM-potassium phosphate buffer, pH 7.0, 0.2 μg of purified enzyme and 4-methyl-2-enelactone in concentrations within the range of 0.1–1 mM. Double-reciprocal and Eadie–Hofstee plots were linear throughout this range, and regression analysis of the data ($r \geqslant 0.990$) gave an apparent K_m of 0.23 mM and a $V_{max.}$ of 530 μmol·min^{-1}·mg of protein^{-1}. Different preparations of the enzyme rarely diverged more than 10% from these values, though there was a slow loss of activity on prolonged storage at −30 °C. The initial rates of conversion of the 1-methyl-bislactone were also measured spectrophotometrically at 212 nm using reaction mixtures with all the components at the concentration of the standard assay. The Michaelis constant obtained for the 1-methyl-bislactone was 0.17 mM, and the $V_{max.}$ was 600 μmol·min^{-1}·mg of protein^{-1}.

Substrate analogues and inhibitors of the isomerase

The ability of various lactone analogues to serve as substrates was investigated by replacing (+)-4-methyl-2-enelactone with each analogue at a concentration of 0.3 mM in a reaction mixture containing 50 mM-potassium phosphate buffer, pH 7.0, and 0.3 μg of purified isomerase in a final volume of 1 ml. The concentration of substrate used in these assays was conditioned by (i) the small amounts of **I** originally available and (ii) its molar absorption coefficient at 212 nm, which, at concentrations above 0.6–0.7 mM, gave A_{210} values approaching

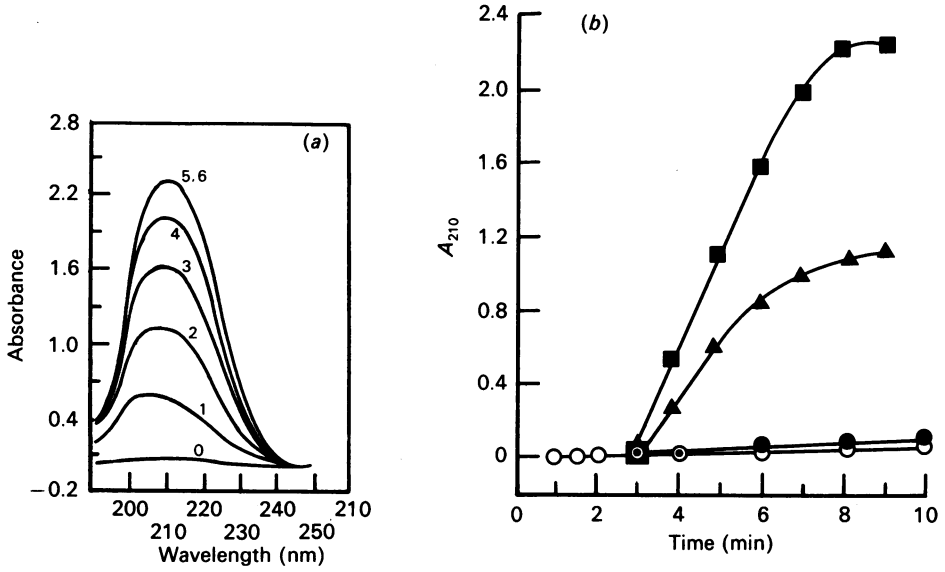


Fig. 6. Enzymic conversion of 1-methylbislactone into 3-methyl-2-enelactone

(*a*) Spectral changes resulting from the addition of purified enzyme (0.3 μg of protein) at time zero to a solution containing 0.3 μmol of 1-methylbislactone in 1 ml of 50 mM-potassium phosphate buffer, pH 7.0. Spectra were scanned initially (0) then, after adding enzyme, at intervals of 1 min as indicated. (*b*) Time course of the delactonization of 0.3 μmol of (−)-1-methylbislactone (■), and the corresponding racemic ± compound (▲) in 50 mM-potassium phosphate buffer, pH 7.0, after addition of the isomerase (0.3 μg of protein) at 3 min. ○, Rate in the absence of enzyme; ●, after addition of boiled enzyme (0.3 μg of protein) at 3 min.

3.0, at the limits of instrument sensitivity. In a parallel series, 4-methyl-2-enelactone at 0.3 mM was also added to reaction mixtures that contained a 0.6 mM concentration of an analogue compound to ascertain whether any of them acted as an inhibitor of the interconversion of the 4-methyl- into the 3-methyl-2-enelactone. Table 2 lists the substrate analogues tested and the standard assay system employed. For compounds showing significant inhibition of the enzyme, K_i values for the inhibitors were measured (Table 2) over a range of substrate and inhibitor concentrations by the h.p.l.c. or spectrophotometric method as appropriate, but most analogues caused only marginal (less than 20%) inhibition when present at twice the substrate concentration and were not examined further.

The isomerase demonstrated a high degree of substrate specificity; other than the (+)-4-methyl-2-enelactone, only two of a wide range of analogues acted as substrates, namely the (−)-1-methyl-bislactone (**Va**), but not its (+)-enantiomer, and the synthetic (±)-muconic bislactone (**VI**). Fig. 5 illustrates the structural features of these two compounds, both of which differ from 4-methyl-2-enelactone in possessing two lactone bridges forming a fused difuran structure. They had not previously been known to be biologically active, even though they had been isolated from biological sources (Catelani *et al.*, 1971).

The relative rate of activity of the purified enzyme from *R. rhodocrous* with the (−)-1-methylbislactone (**Va**) and the bislactone (**VI**) as substrates were respectively 2.2- and 3.8-fold higher than that determined with 4-methyl-2-enelactone itself. The (−)-isomer of **Va** was converted into **II** approximately twice as fast as the racemic (±) compound and gave twice the yield of **II** (0.98 and 0.55 mol of **II** per mol of 1-methylbislactone); the (+)-isomer of methylbislactone is thus biologically inactive. The bromo derivative (**Vb**), by contrast, very rapidly hydrolysed non-enzymically in phosphate buffer, pH 7.0. The hydrolysis product has been identified by Dr. G. V. Rao as 2-bromo-3-methylmuconolactone. It was also only a very weak inhibitor of the enzymic delactonization of **Va** and did not act as an anticipated irreversible inhibitor even when preincubated with the enzyme for 15 min. Both reaction products of the bislactones had characteristic u.v. absorbance peaks, at 212 nm for the 1-methylbislactone product (Fig. 6) and 207 nm for the product of the unsubstituted bislactone. These reaction mixtures were examined by h.p.l.c. using authentic samples of 4-methyl- and 3-methyl-2-enelactone and muconolactone as standards. The single reaction product of the enzyme on 1-methylbislactone had a retention time coincident with that of 3-methyl-2-enelactone, whereas the retention time of the reaction product of the enzyme on the muconic bislactone (**VI**) coincided with that for muconolactone. Both bislactones were unstable in phosphate buffer, pH 7.0, and over several hours slowly decomposed, the unsubstituted bislactone (**VI**) to muconolactone and the 1-methyl-bislactone (**Va**) to a mixture of 3-methyl- and 4-methyl-2-enelactones. The rate of enzymic degradation of the two bislactones (Fig. 6*b*) by 0.3 μg of purified isomerase was, however, at least 20-fold higher than their non-enzymic rates of hydrolysis. Enzymic activity by the h.p.l.c. assay could not be detected with muconolactone, which in any case lacks a methyl substituent, so substrate and putative product are identical. It acted, however, as a good competitive inhibitor of the conversion of both 4-methyl-2-enelactone and (−)-1-methylbislactone to 3-methyl-2-enelactone, (K_i = 0.48 mM and 0.65 mM respectively), as measured by the h.p.l.c. assay. 3-Carboxymuconolactone was not a substrate for the isomerase, nor was it an inhibitor of the utilization of **I** by the enzyme.

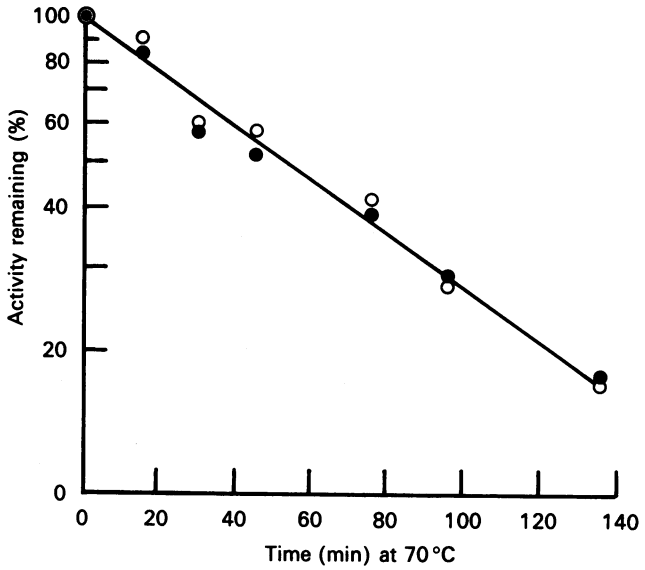


Fig. 7. Time course of heat inactivation of 4-methyl-2-enelactone methyl-isomerase

Purified samples of enzyme were incubated at 70 °C in 50 mM-potassium phosphate buffer, pH 7.0. Activities were determined as described in the Materials and methods section, with 0.3 mM-4-methyl-2-enelactone (○) and 0.3 mM-1-methylbislactone (●).

Thermal denaturation of the purified enzyme indicated that the enzymically catalysed conversion of 4-methyl-2-enelactone and 1-methylbislactone into 3-methyl-2-enelactone had the same half-life (54 min) at 70 °C (Fig. 7) and was thus probably catalysed by the same active centre.

Table 3. Effect of inhibitors on the 4-methyl-2-enelactone methyl-isomerase activity

A sample of pure enzyme (0.3 μg) was preincubated for 10 min at 30 °C in 50 mM-potassium phosphate buffer, pH 7.0, in the presence of the reagent indicated before the addition of 0.3 μmol of 4-methyl-2-enelactone. Re-activation with dithiothreitol was tested in the mixture with enzyme and *p*-chloromercuribenzoate after 15 min incubation of all the components. The absolute activity, using 0.3 μg of enzyme, was 0.09 μmol·min^{-1} (≡ 100 %).

Addition to the assay mixture	Concn. (mM)	Relative enzyme activity (%)
None	–	100
$AgNO_3$	1	14
$HgCl_2$	1	5
$CuSO_4$	1	1
KCN	1	100
p-Chloromercuribenzoate	0.01	10
	0.05	0
p-Chloromercuribenzoate + dithiothreitol	0.05 + 10	38
Dithiothreitol	1	117
EDTA	1	100
8-Hydroxyquinoline	1	95

Effect of heavy metals, chelating agents, thiol reagents and reducing reagents on the isomerase activity

The activity of the enzyme remained practically unchanged upon prolonged dialysis against 50 mM-potassium phosphate buffer, pH 7.0. Chelating agents, such as EDTA, were also without effect on enzyme activity, and the isomerase showed no significant requirement for the usual bivalent-metal ions. Heavy-metal ions inhibited the enzyme almost completely, suggesting the involvement of thiol groups. The heavy-metal derivative *p*-chloromercuribenzoate, which brings about highly specific modifications of cysteine side chains in enzymes, had a completely inhibitory effect at 0.05 mM on the isomerase, the activity of which was partially protected from this inhibitor by addition of excess thiol (Table 3).

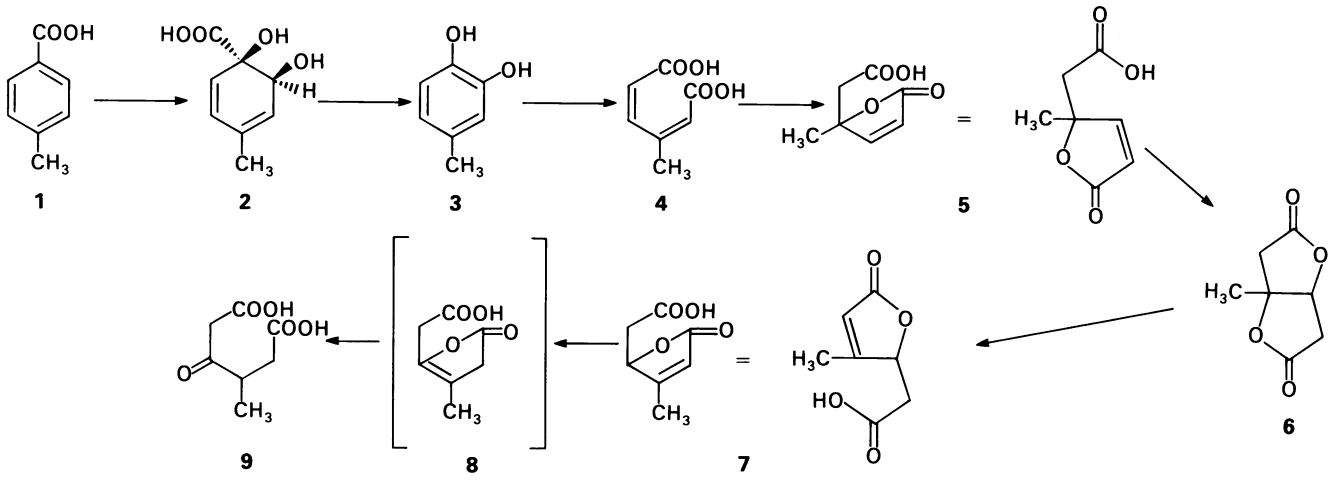
DISCUSSION

It had previously been thought that the only metabolic route by which bacteria could degrade methyl-substituted benzoates was via the well-studied *meta*-cleavage pathway (Murray *et al.*, 1972; Sala-Trepat *et al.*, 1972; Williams & Murray, 1974; Bayly & Barbour, 1984), because, in species of the genus *Pseudomonas*, dissimilation of methyl-substituted aromatics by an *ortho*-cleavage route afforded the 'dead-end' metabolite 4-methyl-2-enelactone (Catelani *et al.*, 1971; Knackmuss *et al.*, 1976). This product accumulates because, in aromatic catabolism, enzymic delactonization of (+)-muconolactone involves first a double-bond isomerization, initiated by a shift of the proton at C-4 to C-2, resulting in the formation of 3-oxoadipate enol-lactone (Stanier & Ornston, 1973); a methyl substituent at C-4 would leave no free proton to undergo such a shift. Extracts from *R. rhodocrous* and other actinomycetes, however, contain a novel enzyme which has the property of catalysing the conversion of 4-methyl-2-enelactone into its 3-methyl isomer; this intermediate is then further metabolized (Bruce & Cain, 1988). A similar modified *ortho*-fission pathway has also been described for a wild-type strain of *Alcaligenes eutrophus* JMP 134 (Pieper *et al.*, 1985).

In contrast, the eukaryotic organisms *Trichosporon cutaneum* (Powlowski & Dagley, 1985), *Aspergillus niger* and *Penicillium thomii* (G. W. Cameron & R. B. Cain, unpublished work) convert 3-methyl-*cis,cis*-muconate directly into (−)-3-methyl-2-enelactone and were without activity on the isomeric (+)-4-methyl-2-enelactone. Thus the utilization of methylmuconate seems to occur via the 4-methyl-2-enelactone (**I**) intermediate exclusively in bacteria, even though some genera lack the ability to metabolize this lactone further.

The novel enzyme, 4-methyl-2-enelactone methyl-isomerase, purified 215-fold to electrophoretic and h.p.l.c. homogeneity, is responsible for further metabolism of **I** in those genera which are genetically competent to do so. The isomerase in rhodococci was a probable tetramer and was found to be very thermostable, a property that enabled a large fraction of the contaminating protein to be removed by denaturation at 65 °C. Furthermore, the purified enzyme was found to be highly specific with respect to the interconversion of its substrates, but its reaction mechanism is the feature of most interest.

Significantly, the purified enzyme exhibited no activity towards either *cis,cis*- or the *cis,trans*-isomers of 3-



Scheme 1. Proposed pathway for the dissimilation of *p*-toluate in nocardioform actinomycetes

1, *p*-toluate; **2**, *p*-toluate-*cis*-hydrodiol; **3**, 4-methylcatechol; **4**, 3-methyl-*cis*,*cis*-muconate; **5**, 4-methyl-2-enelactone; **6**, 1-methyl-2-enelactone; **7**, 3-methyl-2-enelactone; **8**, 3-methyl-3-enelactone (4-carboxymethyl-3-methylbut-3-en-1,4-olide; postulated as an intermediate by analogy with 3-oxoadipate enol lactone); **9**, 4-methyl-3-oxoadipate.

methylmuconic acid; neither were they effective competitive inhibitors, showing that it is unlikely that the interconversion of the 4-methyl- to the 3-methyl-2-enelactone first involved ring opening, isomerization of the resulting 3-methyl-*cis*,*cis*-muconate (**XIV**) to the 3-methyl-2-*cis*-4-*trans*-muconate (**XV**) and relactonization of the latter (Catelani *et al.*, 1971). The enzymic mechanism thus apparently requires a *formal* shift of a methyl group on the lactone ring, an isomerization reaction which has not previously been described in microbial aromatic metabolism, though shifts of methyl groups (Nametkin and Wagner–Meerwein rearrangements) are well known in the chemistry of the steroids (Dutler *et al.*, 1957; Blunt *et al.*, 1966) and the terpenoids (Templeton, 1969) and have been observed with aliphatic compounds (Sykes, 1970).

The *formal* shift catalysed by the new isomerase involves conversion of 4-methyl-2-enelactone (**I**), which already has a tertiary centre at C-4 and an unsubstituted double bond, into the 3-methyl-2-enelactone (**II**) with a more highly substituted double bond. The latter alkene is chemically the more stable isomer (Sykes, 1970), which accounts for the fact that the enzymic reaction is virtually unidirectional and favours quantitative formation of the 3-substituted lactone (**II**). Nevertheless, the reaction is an isomerization and could fall into the E.C. category 5.4.99.- (transferases or mutases; in which the overall reaction is equivalent to the intramolecular transfer of a methyl group from one position to another) or 5.5.1.-. (intramolecular lyases or cycloisomerases; in which the methyl group is eliminated from one part of the molecule, C-4, leaving a double bond, while remaining covalently attached to the molecule, at C-3, in the final product) (IUB, 1984). Our putative reaction mechanism (Scheme 1, compound 6) implicates a cycloisomerization step and does not involve actual methyl migration (see below) although the *overall* reaction has the net result of interconverting the 4-methyl and 3-methyl isomers. We propose the systematic name '4-carboxymethyl-4-methylbut-2-en-1,4-olide:4-carboxymethyl-3-methylbut-2-en-1,4-olide methyl-isomerase' (trivially, 4-methylmuconolactone methyl-isomerase) for the enzyme, describing its overall reaction, until the mechanism is further clarified.

The reaction substrate (**I**) (Catelani *et al.*, 1971; Knackmuss *et al.*, 1976) and the analogous (+)-muconolactone (**IV**) (Avigad & Englard, 1969) are both dextrorotatory and share the same absolute configuration (4*S*) at C-4 (Cain *et al.*, 1989). The product (**II**) of the methyl-isomerase reaction is, in contrast, laevorotatory, in which it resembles (−)-3-carboxymuconolactone (**XIII**) (Kirby *et al.*, 1975), the intermediate formed from 3-carboxy-*cis*,*cis*-muconate by many fungi (Gross *et al.*, 1956; Cain *et al.*, 1968). The unexpected inversion of optical rotation in the methyl-isomerase reaction prompted an investigation of the stereochemistry of the reaction. The absolute stereochemistry of the biologically active (−)-3-methyl-2-enelactone (**II**) product has recently been determined (Cain *et al.*, 1989) and is identical with that of the substrate (**I**), of (+)-muconolactone (**IV**) and of (−)-3-carboxymuconolactone (**XIII**), all of which are 4*S* (Fig. 8). Only 4-carboxymuconolactone (**XVI**), arising from eubacterial lactonization of 3-carboxymuconate, has the opposite 4*R* configuration (Chari *et al.*, 1987). The unexpected optical configuration of biologically active compound **II** was not an (albeit unlikely) artifact of the isolation procedure; when the racemic (±) compound **II** was incubated with extracts of *R. rhodocrous*, only the (−)-isomer was utilized (Bruce & Cain, 1988).

Nametkin and Wagner–Meerwein methyl rearrangements involve the formation of a carbonium-ion intermediate and a shift of the methyl group to this cationic centre with, in appropriate circumstances, inversion of configuration (Hartshorn & Kirk, 1966). The maintenance of the absolute configuration in the methyl-isomerase reaction is more commensurate with a mechanism in which the *formal* methyl shift of the isomerase reaction involves no actual methyl migration but proceeds *via* the formation of 1-methylbislactone (**Va**) from **I** with subsequent opening of the opposite furan ring to form **II** (see Scheme 1, compounds **5**–**7**). It is significant that, of all the analogues tested, the 1-methylbislactone (**Va**) and its simple muconic bislactone analogue (**VI**) were the only other substrates utilized by the enzyme,

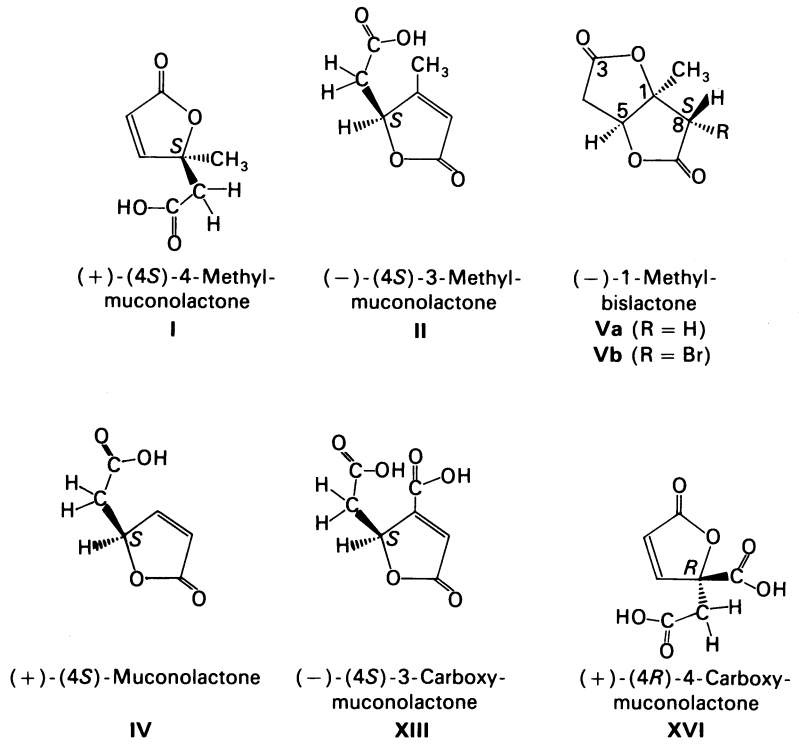


Fig. 8. Stereochemical configurations of the biologically active isomers and analogues of the muconolactones

being converted into 3-methyl-2-enelactone and muconolactone respectively.

The likely involvement of a bislactone in aromatic catabolism was first proposed by Elsden & Peel (1958) to account for the randomization of the ^{14}C label during the catabolism of [2,6-$^{14}C_2$]protocatechuate by fungi. Such bislactones were subsequently isolated from cultures of *Oospora* incubated with catechol (Landa & Eliasek, 1956) and from *Pseudomonas desmolyticum* incubated with 4-methylcatechol (Catelani *et al.*, 1971), but both groups of authors considered them artifacts of chemical-isolation procedures, even though the (−)-1-methylbislactone (**Va**) isolated by Catelani *et al.* (1971) was optically active; this feature arose because this bislactone has two asymmetric centres. Cain *et al.* (1961) had observed that the ring of muconic bislactone (**VI**) was opened by extracts of protocatechuate-grown bacteria at rates only marginally faster than that of its chemical hydrolysis, but synthesis of **Va** from **I** and the subsequent enantiomeric resolution from the mixture by Dr. G. V. Rao of the corresponding (−)-1-methylbislactone (**Va**), now known to have the absolute stereochemistry shown in Fig. 8 (Cain *et al.*, 1989), prompted an examination of its utilization by the purified isomerase by which it was rapidly hydrolysed some 20-fold faster than the non-enzymic rate of hydrolysis (Fig. 6*b*). The specific activity of the purified isomerase with (−)-1-methylbislactone was greater than that observed with 4-methyl-2-enelactone, making it unlikely that the bislactone would be readily detected as a reaction intermediate, yet the K_m values for both substrates and the K_i values for their competition by (+)-muconolactone were very similar, suggesting that they were bound to the enzyme equally efficiently. It is significant that a charged group at C-3 (**XIII**), however, renders (+)-muconolactone an ineffective inhibitor. The stereochemical configuration of **Va**, with its now confirmed *cis*-fusion of the two furan rings (Fig. 8), is also commensurate with its participation as an intermediate in the overall reaction. Experiments with deuterium-labelled substrates currently underway in *Rhodococcus* should show whether lactone-ring closure and opening occurs in a *syn* fashion as it does in *Pseudomonas* and *Aspergillus* (G. V. Gao, G. W. Kirby & R. B. Cain, unpublished work).

It is equally possible, however, that the putative 1-methylbislactone intermediate (**Va**) may occur, in the overall isomerization, only as an enzyme-bound transition-state intermediate, perhaps linked to a thiol group at the active site. The involvement of such a residue is suggested by the extreme sensitivity of the enzyme to heavy metals and thiol reagents (Table 3). Such an intermediate would be impossible to detect as a free product by isotope-trapping techniques.

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REFERENCES

Ahlquist, E. R. (1977) Ph.D. Thesis, University of Kent
Ainsworth, A. T. & Kirby, G. W. (1968) J. Chem. Soc. C, 1483–1487
Andrews, P. (1964) Biochem. J. **91**, 222–223

Avigad, G. & Englard, S. (1969) Fed. Proc. Fed. Am. Soc. Exp. Biol. **28**, 345–347
Bayly, R. C. & Barbour, M. G. (1984) in Microbial Degradation of Organic Compounds (Gibson, D. T., ed.), pp. 253–294, Marcel Dekker, New York
Blunt, J. W., Hartshorn, M. P. & Kirk, D. N. (1966) Tetrahedron **22**, 3195–3202
Bruce, N. C. & Cain, R. B. (1988) FEMS Microbiol. Lett. **50**, 233–239
Cain, R. B., Ribbons, D. W. & Evans, W. C. (1961) Biochem. J. **79**, 312–316
Cain, R. B., Bilton, R. F. & Darrah, J. A. (1968) Biochem. J. **108**, 797–827
Cain, R. B., Freer, A. A., Kirby, G. W. & Rao, G. V. (1989) J. Chem. Soc. Perkin Commun. 202–203
Catelani, D., Fiecchi, A. & Galli, E. (1971) Biochem. J. **121**, 89–92
Chari, R. V. J., Whitman, C. P., Kozarich, J. W., Ngai, K.-L. & Ornston, L. N. (1987) J. Am. Chem. Soc. **109**, 5514–5519
Dutler, H., Bosshard, H. & Jeger, O. (1957) Helv. Chim. Acta **40**, 494–498
Elsden, S. R. & Peel, H. J. (1958) Annu. Rev. Microbiol. **12**, 145–194
Elvidge, J. A., Linstead, R. P., Orkin, B. A., Sims, P., Baer, H. & Pattison, D. B. (1950) J. Chem. Soc. 2228–2235
Elvidge, J. A., Linstead, R. P. & Sims, P. (1951) J. Chem. Soc. 3386–3397
Gross, S. R., Gafford, R. D. & Tatum, E. L. (1956) J. Biol Chem. **219**, 781–790
Hartshorn, M. P. & Kirk, D. N. (1966) Tetrahedron Lett. 565–567
IUB (1984) Enzyme Nomenclature, 3rd edn., pp. 422–426, Academic Press, London
Knackmuss, H.-J., Hellwig, M., Lackner, H. & Otting, W. (1976) Eur. J. Appl. Microbiol. **2**, 267–276
Kirby, G. W., Loughlin, G. T. & Robbins, D. J. (1975) J. Chem. Soc. 402–403
Landa, S. & Eliasek, J. (1956) Chem. Listy. **50**, 1834–1836
Laemmli, U. K. (1970) Nature **227**, 680–682
Miller, D. J. (1981) in Actinomycetes (Zentralbl. Bakteriol. Parasitenk. Infektion Suppl. 11) (Schaal, K. P. & Pulverer, G., eds.), pp. 353–360, Gustav Fischer Verlag, Stuttgart
Murray, K., Duggleby, C. J., Sala-Trepat, J. M. & Williams, P. A. (1972) Eur. J. Biochem. **28**, 301–310
Pieper, D. H., Engesser, K.-H., Don, R. H., Timmis, K. N. & Knackmuss, H.-J. (1985) FEMS Microbiol. Lett. **29**, 63–67
Powlowski, J. B. & Dagley, S. (1985) J. Bacteriol. **163**, 1126–1135
Sala-Trepat, J. M., Murray, K. & Williams, P. A. (1972) Eur. J. Biochem. **28**, 347–356
Stanier, R. Y. & Ornston, L. N. (1973) Adv. Microbial Physiol. **9**, 89–151
Sykes, P. (1970) A Guide Book to Mechanism in Organic Chemistry, pp. 96–118, Longman, London
Templeton, W. (1969) An Introduction to the Chemistry of the Terpenoids and Steroids, pp. 1–24, Butterworth, London
Williams, P. A. & Murray, K. (1974) J. Bacteriol. **134**, 757–764

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